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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Breast tumor kinase (Brk), a novel non-receptor protein tyrosine kinase cloned from a metastatic human breast tumor (1), is overexpressed in approximately two-thirds of human breast tumors, but is absent from normal breast epithelial cells (2). Little is known about the role of Brk in breast cancer. Data from our lab has shown that shRNA knockdown of endogenous Brk in T47D breast cancer cells blocked ERK5 and p38 mitogen activated protein kinase (MAPK) activation in the presence of heregulin and EGF. Brk gene-silencing also induced T47D cell growth inhibition and reduced migration in Boyden chamber assays relative to control (non-specific shRNA) conditions. We have preliminarily shown that Brk kinase activity appears to be required for p38 MAPK and Erk5 phosphorylation, and that Brk protein appears to have a long half-life. These results are preliminary, but are beginning to approach areas of Brk related research that have not been addressed.

15. SUBJECT TERMS
Brk/ptk6, Erk5, p38, MAPK, breast cancer, ErbB signaling

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Introduction

Breast tumor kinase (Brk), a novel non-receptor protein tyrosine kinase cloned from a metastatic human breast tumor (1), is overexpressed in approximately two-thirds of human breast tumors, but is absent from normal breast epithelial cells (2). Little is known about the role of Brk in breast cancer. Data from our lab has shown that shRNA knockdown of endogenous Brk in T47D breast cancer cells blocked ERK5 and p38 mitogen activated protein kinase (MAPK) activation in the presence of heregulin and EGF. Brk gene-silencing also induced T47D cell growth inhibition and reduced migration in Boyden chamber assays relative to control (non-specific shRNA) conditions.

The role of Brk in driving proliferation and survival of breast cancer cell lines will be investigated. It is my hypothesis that Brk is signaling to ERK5 and p38 MAPK, thus conferring a survival signal that allows changes in cell biology (i.e. anchorage independent growth, evasion of apoptosis, and/or increased migration) that are responsible for breast cancer progression in tumors overexpressing Brk. The overall aims of this project are outlined in figure 1.

Figure 1. Overview of Aims and Signaling Model
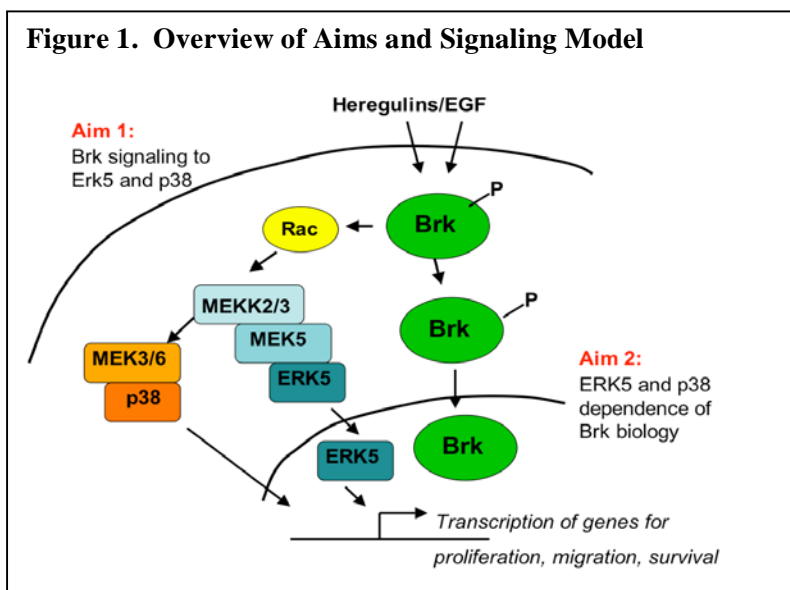
Body

Specific Aim 1: What is the specificity of Brk signaling to the Erk5 and p38 MAPK signaling pathways?

1a) Are MEKK2/3 and MEK3/6 activated in response to ErbB ligand induced Brk signaling?

1b) Is Brk "downstream" signaling to Erk5 and p38 in breast cancer cells dependent on activation of MEKK2/3 and/or MEK3/6 MAPK-modules?

These tasks have not been done directly as listed in the grant proposal, due to technical difficulty with our cell-based system. Once remedied, these tasks will be completed as outlined in the grant.

As mentioned in the grant proposal for this fellowship and in Ostrander, et al (3, and Appendix A), we have stably expressed Brk specific shRNA in breast cancer cell lines that endogenously express Brk. These cells were going to be the model system used for specific aim 1. However,

the levels and consistency of Brk knockdown has been difficult to maintain. After a small number of passages in culture, the inhibition of Brk expression decreases considerably. Additionally, pooled cultures with strong Brk knockdown at the time of their development appear to lose their degree of knockdown upon thawing them from liquid nitrogen storage. I have used the same stable transfection method (RetroQ from Clontech) to establish new Brk knockdown lines, however instead of pooling successfully transfected cells, I have developed clonal populations of Brk knockdown cells as a variation on the system. As illustrated in Ostrander et al, T47D breast cancer cells exhibit decreased proliferation when Brk expression is inhibited. Therefore, by pooling knockdown cells during the selection process, it is thought that the cells with a low inhibition of Brk expression proliferate faster than the cells with strong Brk knockdown, in turn decreasing the overall level of Brk knockdown over time. One potential concern is that clones with strong Brk knockdown will not proliferate enough to be maintained in culture. This is currently being evaluated.

Brk protein stability may also be an explanation for the difficulty in maintaining Brk knockdown cell lines. To investigate the half-life of Brk, T47D breast cancer cells were treated with 10 uM

cycloheximide (a protein synthesis inhibitor) for eight hours, lysed, and subjected to SDS-PAGE and Western blotting (fig. 2). The results that we have seen indicate two possible situations. One, Brk protein has a half-life of greater than eight hours, or two the cycloheximide is not working. A timecourse of various treatment times and concentrations will be done to determine


Figure 2. Brk Protein Stability in T47D Cells
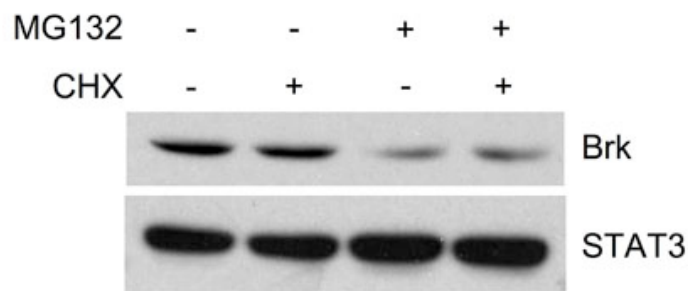
Western blot of T47D cells treated with MG132 or cycloheximide (CHX). Total STAT3 was used as a loading control.

In addition, SUMOplot analysis (www.abgent.com/doc/sumoplot) of the amino acid sequence for Brk revealed the presence of putative SUMOylation motifs. SUMOylation of proteins could inhibit the ubiquitination and subsequent proteasomal degradation of the target protein (4). To determine if Brk undergoes proteasome-mediated degradation, T47D breast cancer cells were treated with for eight hours with 10 uM MG132, a proteasome inhibitor (fig. 2) and eight hours with 10uM cycloheximide. The cells were then lysed and subjected to SDS-PAGE and Western blotting with a Brk specific antibody. If Brk undergoes proteasomal degradation, we would expect to see an increase in Brk expression in lane 3 relative to the Brk level in lane 1. Also, an increase in Brk expression was expected in lane 4 relative to lane 3. We did not see this; it may be due to the inhibitors being ineffective (incorrect concentration or treatment length). This experiment has only been done once as shown in fig. 3, and will be repeated with additional treatment conditions.

To confirm that the differential p38 MAPK phosphorylation we saw in T47D cells with Brk knockdown was a valid result, we overexpressed wild-type (wt), kinase dead (KM), and

constitutively active (YF) Brk in parental T47D cells. We transiently transfected Flag-tagged Brk constructs that were previously made in the lab, in order to differentiated between endogenous and overexpressed Brk. Cells were treated as in Ostrander, et. al., lysed, and subjected to Western blotting with phospho-p38 antibodies. As shown in figure 3, it appears that p38 phosphorylation is dependent on Brk kinase activity, further validating the results in Ostrander, et. al.

Figure 3. Brk Activity Mediates MAPK Activation
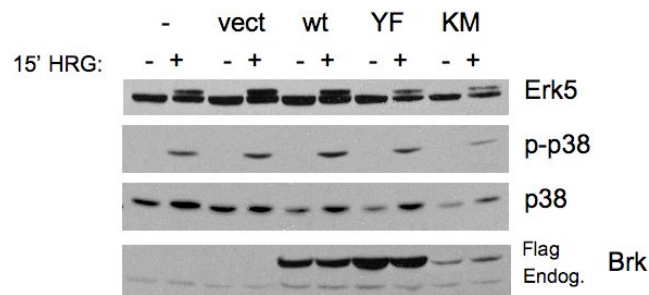
Western blot of T47D cells transiently transfected with Brk expression vectors. IB for Erk5 and p38 MAPKs.

Specific Aim 2: What are Brk-induced changes in breast cancer biology?
2a) Is anchorage independent growth of breast cancer cells dependent on heregulin induced Brk signaling?

These studies have not been initiated. These experiments rely on having stable Brk-knockdown cells. Once the knockdown issue mentioned above is resolved, these studies will begin.

2b) Is the survival of Brk-positive breast cancer cell lines in the presence or absence of chemotherapeutic agents enhanced by Brk-induced Erk5 and/or p38 MAPK activity?

These studies have not been initiated. These experiments also rely on stable knockdown of Brk protein expression.

Key Research Accomplishments

- Heregulin-mediated Erk5 and p38 MAPK phosphorylation in breast cancer cell lines appears dependent on Brk kinase activity.
- Brk contains a putative SUMOylation sequence in its kinase domain

Reportable Outcomes

Publications

Ostrander JH, Daniel AR, Lofgren K, Kleer CG, Lange CA. Breast Tumor Kinase (Protein Tyrosine Kinase 6) Regulates Heregulin-Induced Activation of ERK5 and p38 MAP Kinases in Breast Cancer Cells. Cancer Research 67(9):4199-4209, 2007.

Presentations

Lofgren KA, Lange CA. Brk Mediated Signaling and Mammary Gland Biology Relevant to Breast Cancer. 2007 Gordon Research Conference: Mammary Gland Biology, Newport, RI, June 2007. *Oral Presentation.*

Lofgren KA, Ostrander JH, Vanasek T, Qiu M, Lange CA. Breast Tumor Kinase Mediates Signaling and Biology Relevant to Breast Cancer Tumorigenesis and Progression. 2007 Gordon Research Conference: Mammary Gland Biology, Newport, RI, June 2007. *Poster Presentation.*

Lofgren KA, Ostrander JH, Vanasek T, Qiu M, Lange CA. Breast Tumor Kinase Mediates Signaling and Biology Relevant to Breast Cancer Tumorigenesis and Progression. University of Minnesota Medical Research Day, June 2007. *Poster Presentation.*

Conclusion

We hypothesize that Brk mediates heregulin induced growth factor signaling that promotes the malignant phenotype in breast cancer cells. We have shown that Erk5 and p38 MAPKs are activated by Brk kinase activity, confirming our hypothesis that malignant phenotypes are mediated through this pathway. Once the model system of Brk-knockdown cells is improved, future studies will determine which signaling molecule is controlling the malignant phenotype, as outlined in the grant.

Signficance
Protein tyrosine kinases act as regulators of normal cell differentiation, proliferation and apoptosis; elevated protein tyrosine kinase activity levels are reported in many invasive breast cancers and is indicative of a poor prognosis (5,6). In the search for new therapeutic targets, the actions of protein tyrosine kinases like Brk are of high interest. As Brk is not expressed in the normal mammary gland, targeting Brk in breast cancers is predicted to be a highly specific approach. Through the investigation of Brk and Brk-mediated events in cancer, not only can a better understanding of breast cancer be achieved, but it may also promote the potential of using Brk-targeted therapeutics.

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3. Ostrander JH, Daniel AR, Lofgren K, Kleer CG, Lange CA. Breast Tumor Kinase (Protein Tyrosine Kinase 6) *Regulates Heregulin-Induced Activation of ERK5 and p38 MAP Kinases in Breast Cancer Cells.* Cancer Research, 2007. **67**(9):p.4199-4209.

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Breast Tumor Kinase (Protein Tyrosine Kinase 6) Regulates Heregulin-Induced Activation of ERK5 and p38 MAP Kinases in Breast Cancer Cells

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Abstract

Total tyrosine kinase activity is often elevated in both cytosolic and membrane fractions of malignant breast tissue and correlates with a decrease in disease-free survival. Breast tumor kinase (Brk; protein tyrosine kinase 6) is a soluble tyrosine kinase that was cloned from a metastatic breast tumor and found to be overexpressed in a majority of breast tumors. Herein, we show that Brk is overexpressed in 86% of invasive ductal breast tumors and coexpressed with ErbB family members in breast cancer cell lines. Additionally, the ErbB ligand, heregulin, activates Brk kinase activity. Knockdown of Brk by stable expression of short hairpin RNA (shRNA) in T47D breast cancer cells decreases proliferation and blocks epidermal growth factor (EGF)- and heregulin-induced activation of Rac GTPase, extracellular signal-regulated kinase (ERK) 5, and p38 mitogen-activated protein kinase (MAPK) but not Akt, ERK1/2, or c-Jun NH$_2$-terminal kinase. Furthermore, EGF- and heregulin-induced cyclin D1 expression is dependent on p38 signaling and inhibited by Brk shRNA knockdown. The myocyte enhancer factor 2 transcription factor target of p38 MAPK and ERK5 signaling is also sensitive to altered Brk expression. Finally, heregulin-induced migration of T47D cells requires p38 MAPK activity and is blocked by Brk knockdown. These results place Brk in a novel signaling pathway downstream of ErbB receptors and upstream of Rac, p38 MAPK, and ERK5 and establish the ErbB-Brk-Rac-p38 MAPK pathway as a critical mediator of breast cancer cell migration. [Cancer Res 2007;67(9):4199–209]

Introduction

A key step in the progression of a majority of steroid hormone receptor–positive breast cancers is the transition from steroid hormone-dependent to steroid hormone-independent proliferation. Estrogen-independent and tamoxifen-resistant tumors often have increased levels of the ErbB family of receptor tyrosine kinases. In addition to overexpression of ErbB family members, total tyrosine kinase activity is often enhanced in both cytosolic and membrane fractions of malignant breast tissue and correlates with a decrease in disease-free survival. Breast tumor kinase (Brk) is a soluble tyrosine kinase that was cloned from a metastatic breast tumor. Although Brk is undetectable in the normal mammary gland, it is overexpressed in at least 65% of human breast tumors and breast cancer cell lines, with highest levels in advanced tumors (1, 2). Brk overexpression also occurs in colon tumors and melanoma cancer cell lines. Brk is differentially localized in prostate cancer cells relative to the normal gland (3–5).

Although Brk is ∼56% homologous to c-Src within the kinase domain and similarly encodes a "soluble" tyrosine kinase with tandem NH$_2$-terminal SH3 and SH2 domains, Brk is a member of a novel family of soluble protein tyrosine kinases, considered to be distantly related to c-Src (6). In contrast to Src family kinases, Brk does not contain an NH$_2$-terminal myristoylation site, suggesting that Brk is not tightly associated with the plasma membrane. In fact, Brk localizes to the nucleus of some breast and prostate cancer cell lines (4). Additionally, a recent report suggests that the Brk SH2 domain is structurally different than most SH2 domains and does not have a high affinity for the proposed autoinhibitory tyrosine of Brk (Y447; ref. 7). These results suggest that Brk may be regulated differently than other Src family tyrosine kinases and/or that Brk may interact with novel signaling pathways.

Several Brk substrates and interacting proteins have been identified (8). The first reported substrate for Brk phosphorylation in vivo was Sam68. Recently, the Sam68-like proteins, SLM-1 and SLM-2, were identified as Brk substrates (9). Similar to Sam68, phosphorylation of SLM-1 and SLM-2 by Brk leads to inhibition of their RNA-binding function (9). BKS, a novel adaptor-like molecule, which functions to regulate signal transducer and activator of transcription 3 (STAT3) activation (10), has also been shown to interact with and be phosphorylated by Brk. Related to this finding, Liu et al. (11) showed that Brk phosphorylates STAT3. Brk-induced phosphorylation of paxillin was associated with epidermal growth factor (EGF)–induced cellular migration and invasion (12). Additional Brk interacting proteins include IRS-4 and GAP-A.p65 (13, 14). We showed that Brk interacts with Akt in breast cancer cells and human keratinocytes (15). Brk expression induced phosphorylation of Akt on tyrosine residues, and this seemed to limit basal Akt activity in the absence of growth factor stimulation (15). Although Brk substrates have been identified and many of these proteins have been implicated in breast cancer biology, very little is known about the physiologic significance of Brk-induced phosphorylation events and the function of Brk in normal human tissues or during breast cancer progression.

In normal tissues, Brk expression seems to be largely restricted to the differentiating epithelial cells of the gastrointestinal tract and skin (3, 16). Sik (Src-like intestinal kinase), the Brk murine orthologue, is similarly expressed. Sik knockout mice, which initially seemed normal, have been reported recently to display increased proliferation, decreased apoptosis, and activated Akt in the small intestine (17), consistent with Brk-induced inhibition of

Akt signaling in normal tissues (15). In murine keratinocytes, Sik is activated during calcium-induced differentiation (14). Similarly, in human keratinocytes, Brk expression increased in response to differentiation conditions, and calcium/ionomycin induced Brk kinase activity (18). These results suggest a role for Brk in differentiation of epithelial cells of the small intestine and skin.

Interestingly, in breast cancer cells, Brk seems to have an opposite effect on cell growth. Brk potentiates EGF-induced proliferation of MCF-10A and HB4a mammary epithelial cells (19) and knockdown of Brk with RNA interference (RNAi) modestly inhibited the proliferation of T47D cells (20). Additionally, initial studies examining the role of Brk in breast cancer showed that Brk overexpression enhances anchorage-independent growth of the mammary epithelial cell line HB4a and NIH3T3 murine fibroblasts (19).

Additional evidence supports the involvement of Brk in EGF receptor (EGFR)/ErbB receptor signaling. First, Brk has been shown to interact with EGFR and ErbB3 (19, 21). Second, expression of Brk enhances EGF-induced ErbB3 phosphorylation on tyrosine and the recruitment of p85 phosphatidylinositol 3-kinase (PI3K) to ErbB3, which potentiates PI3K activity (21). Third, we reported association of Brk and EGFR following receptor activation in COS1 cells (15). Finally, more recently, a significant correlation between ErbB2 and Brk expression in human breast tumors has been reported (1).

The objective of our current research is to understand how Brk modulates ErbB receptor signaling pathways. Herein, we show that the ErbB3 and ErbB4 ligand, heregulin, activates Brk kinase activity. Furthermore, breast cancer cells expressing Brk short hairpin RNA (shRNA) have reduced Rac, p38, and extracellular signal-regulated kinase (ERK) 5 activation in response to EGF and heregulin, which results in decreased cyclin D1 expression and reduced cell migration. Additionally, we show here for the first time that the myocyte enhancer factor 2C (MEF2C) transcription factor is expressed in normal mammary epithelial cells and in breast cancer cell lines. Brk expression enhances MEF2-luciferase activity and p38 inhibitors block EGF- and heregulin-induced MEF2 activity. Our findings identify novel downstream Brk effector pathways, p38, ERK5, and MEF2 and enhance our understanding of the role of Brk in breast cancer progression. Investigation of Brk and its downstream effectors may provide useful targets for the treatment of advanced metastatic breast cancer.

Materials and Methods

Immunohistochemistry and breast tumor array studies. Immunohistochemistry was done on a previously described human breast tissue microarray (TMA; ref. 22) using standard biotin-avidin complex technique and Brk-specific antibodies characterized for use in immunohistochemistry (Santa Cruz Biotechnology) and Western blotting as described previously (15), using all the reagents except the primary antibody as negative controls and following standard immunohistochemistry procedures. Brk expression was evaluated at least three times for each microarray element and at least nine times for each tumor using a previously validated Web-based tool (TMA Profiler, University of Michigan, Ann Arbor, MI; ref. 23). Brk expression was scored blindly and independently by two pathologists (C.G. Kleer and N. Kirchof, University of Minnesota Histopathology Core) as negative (score, 1), weak (score, 2), moderate (score, 3), and strong (score, 4) based on the intensity of staining and the percentage of tumor cells stained, using a standardized and validated system as described (22, 24). Statistical analysis was done by the University of Minnesota Biostatistics Core facility; the effects of Brk staining intensity on carcinoma content was examined by a linear test for trend using ANOVA.

Cell culture. MCF-10A cells were obtained from the American Type Culture Collection and maintained in 1:1 DMEM/Ham's F-12 supplemented with 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 20 ng/mL EGF, 100 ng/mL cholera toxin, and 5% horse serum. MCF-10A–inducible Brk cell lines were made with Argent Regulated Transcription Retrovirus kit. First, MCF-10A cells were infected with retrovirus encoding two dimerizing transcription factors (pL2N2-RHS3H/ZF3), one encoding a transcriptional activation domain (FRB⁺-p65-HSF1) and the other encoding a DNA binding domain (ZFHD1-3× FKBP). Infected cells were selected for Neo^r and clonal cell populations were screened for expression of dimerizing transcription factors according to the manufacturer's protocol. WT-Brk, KM-Brk, or YF-Brk were subcloned into *Eco*RI and *Cla*1 sites of the inducible retroviral vector pLHZ$_{12}$PL, which is under the control of a promoter containing 12× ZFHD1 binding sites. MCF-10A cells stably expressing dimerizing transcription factors were infected with pLHZ$_{12}$PL-Brk constructs and clonal cell populations were established following selection in hygromycin and screened for inducible Brk expression following the addition of the dimerizer, AP21967. AP21967 is a rapamycin homologue that dimerizes the DNA binding factor and transcriptional activation factor to make a competent transcription factor. Human mammary epithelial cells (HMEC) were a gift from Channing Der (University of North Carolina, Chapel Hill, NC) and maintained in Clonetics MEGM. MCF-7, ZR-75-1, and SKBR3 breast cancer cells were a gift from Doug Yee (University of Minnesota, Minneapolis, MN). MCF-7 cells were maintained in IMEM supplemented with insulin and 5% fetal bovine serum (FBS). ZR-75-1 cells were maintained in RPMI 1640 supplemented with 10% FBS. SKBR3 cells were maintained in McCoy's 5A medium supplemented with 10% FBS. T47D cells were maintained in MEM supplemented with insulin, nonessential amino acids, penicillin/streptomycin, and 5% FBS.

shRNA design and retroviral infection. Three Brk shRNA sequences were generated using BD Biosciences online shRNA oligonucleotide design tool.[3] All sequences generated were BLAST to confirm that no other human genes contained similar sequence homology; those that did not where chosen for further study. These included Brk-A [5′-gatccgtcgcagaattcatc-cacttcaagagagtggatgtaattctgcgacttttttacgcgtg-3′ (sense) and 5′-aattcacgcg-taaaaaagtcgcagaattacatccactctcttgaagtggatgtaattctgcga cg-3′ (antisense)], Brk-B [5′-gatccgctcccatgaccacaatatcttcaagagagatattgtggtcatgggagttttt-tacgcgtg-3′ (sense) and 5′-aattcacgcgtaaaaaactcccatgaccacaatatctctcttgaa-gatattgtggtcatgggagcg-3′ (antisense)], and Brk-C [5′-gatccgtgaccacaatatcccc-tacttcaagagagtaggggatattgtggtcatttt ttacgcgtg-3′ (sense) and 5′-aattcacgcg-taaaaaatgaccacaatatcccctactctcttgaagtaggggatattgtggtcacg-3′ (antisense)]. Sense and antisense oligonucleotides were annealed and ligated into pSIREN RetroQ following the manufacturer's protocol (Clontech). T47D cells were plated at 1×10^5 cells per well in a six-well plate. Twenty-four and 48 h later, 1 mL retrovirus (50% viral supernatant and 50% growth medium) was layered on the cells in the presence of 8 μg/mL polybrene. Forty-eight hours after first infection, cells were trypsinized and replated in 100-mm dishes in normal growth medium containing 1 μg/mL puromycin. After 2 weeks in selection medium, surviving cells were pooled, expanded, and analyzed for Brk protein expression.

Antibodies and reagents. Antibodies to phosphotyrosine (4G10), Rac1 (23A8), cyclin D1, and purified heregulin were from Upstate Biotechnology, Inc. ErbB1 antibody, EGF, and (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma. ErbB2 (Ab-17), ErbB3 (2C3) and ErbB4 antibodies were from Lab Vision. Phosphorylated ERK5 (Thr^{218}/Tyr^{220}), ERK5, phosphorylated p38 (Thr^{180}/Tyr^{182}), total p38, phosphorylated Akt (Ser^{473}), total Akt, phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}), total ERK1/2, phosphorylated c-Jun NH$_2$-terminal kinase (JNK; Thr^{183}/Tyr^{185}), total JNK, and IκBα phosphorylated Ser^{32} antibodies were from Cell Signaling Technology, whereas Brk and IκBα antibodies were from Santa Cruz Biotechnology. Small interfering RNA (siRNA) for cyclin D1 was purchased from Qiagen and transiently transfected into cells with Oligofectamine (Invitrogen). AP21967 and inducible expression system

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[3] http://bioinfo2.clontech.com/rnaidesigner

(Argent Regulated Transcription Retrovirus kit) were obtained from Ariad Pharmaceuticals.

MTT cellular proliferation assay. T47D cells were plated in 24-well plates with 5,000 cells per well in regular growth medium. Growth was measured 1 to 8 days following plating. All time points were measured in triplicate. Growth was assayed by the uptake of MTT as described previously (25).

Brk kinase assay. T47D breast cancer cells were lysed in Brk lysis buffer [BLB; 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mmol/L Na_3VO_4, 20 mmol/L NaF]. For the immunoprecipitation of Brk protein, 1,000 μg total protein was incubated with 1 μg of Brk antibody (preconjugated to Protein G-Agarose beads). To preconjugate the Brk antibody to Protein G-Sepharose beads (Roche), 1 μg Brk or IgG control antibody was incubated with 30 μL Protein G-Sepharose for 1 h at 4°C and then washed three times with BLB. Immunoprecipation reaction was incubated at 4°C for 3 to 4 h. Next, the immunoprecipitation complex was washed with BLB (1 mL) for four times. On the fourth wash, the immunoprecipitation was split into two separate tubes (one for the Brk immunoprecipitation control and one for the kinase reaction). Fifty microliters BLB and 15 μL of 5× Laemmli sample buffer were added to the Brk immunoprecipitation control samples. The kinase reaction samples were then washed with 500 μL of the kinase reaction buffer [100 mmol/L Tris (pH 7.6), 125 mmol/L $MgCl_2$, 25 mmol/L $MnCl_2$, 2 mmol/L EGTA, 0.25 mmol/L Na_3VO_4, 2 mmol/L DTT]. For the kinase reaction, 50 μL of kinase assay buffer with the addition of 100 μmol/L ATP and 2 to 4 μg of purified Sam68 were added to each sample. The kinase reaction was incubated at 30°C for 30 min. To stop the kinase reaction, 15 μL of 5× Laemmli sample buffer were added to each tube. All samples were boiled for 5 min and then separated by SDS-PAGE and transferred to polyvinylidene difluoride for Western blot analysis of tyrosine phosphorylated proteins and Brk.

Rac assay. T47D cells were washed two times in cold 1× PBS and then lysed in buffer B [50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 1% Triton X-100, 0.5 mmol/L $MgCl_2$, supplemented with 1 mmol/L PMSF, 1 μg/mL aprotinin, and 1 μg/mL leupeptin]. One milligram of total protein was then incubated with 30 μg glutathione *S*-transferase (GST)-PAK(PBD) beads for 30 min. The complexes were then washed, and following SDS-PAGE, the amount of GTP-Rac was determined by Western blot using Rac1-specific antibodies. Total cellular lysates were also separated by SDS-PAGE, and Western blot analysis with anti-Rac1 antibodies was done as a control for protein loading.

MEF2-luciferase assay. HeLa, SKBR3, and T47D cells were plated at 2×10^5 cells per well in a six-well plate. Twenty-four hours later, HeLa cells were transfected with 100 ng/well MEF2-luc, 100 ng/well β-actin-LacZ, and indicated amounts of RcCMV-Brk or vector control using Fugene 6 transfection reagent (Roche), whereas SKBR3 and T47D cells were transfected with 1 μg/well MEF2-luc and 20 ng/well pRL-TK (*Renilla* luciferase). The following day, cells were deprived of growth factors overnight and then treated as described in Results. Cells were harvested in 200 μL passive lysis buffer (Promega), and 30 μL of lysate were used to determine relative luciferase, β-galactosidase, or *Renilla* luciferase activity. HeLa cell luciferase activity was normalized to β-galactosidase activity and SKBR3 luciferase activity was normalized to *Renilla* luciferase.

Cell migration assay. Modified Boyden chamber migration assays were done in a 10-well chamber by addition of 0.4 mL serum-free medium (SFM) containing 5 μg/mL collagen I with or without heregulin (25 ng/mL) in the lower chamber. A polycarbonate 12-μmol/L pore membrane (Neuroprobe) was then placed above the lower chamber. T47D cells were trypsinized, washed two times in SFM, and then resuspended at 5×10^5 cells/mL in SFM containing 5 μg/mL collagen I. Cells (0.3 mL) were then placed in the upper compartment and the whole chamber was incubated at 37°C, 5% CO_2 for 6 h. At the end of the incubation, the cells remaining in the upper compartment were removed with cotton swabs. The cells that migrated to the underside of the membrane were fixed and stained with HEMA3. The membrane was then mounted on a glass slide and the cells were counted with the aid of a light microscope.

Results

Brk is overexpressed in invasive breast cancer. Brk protein expression has been reported in a limited number of breast tumor samples and cell lines (2) and was significantly associated with c-ErbB2 (ErbB2) overexpression in 54 archival invasive ductal breast carcinomas (1). To further substantiate the prevalence of Brk overexpression in human breast carcinoma, we examined Brk protein expression in 250 samples of human invasive breast cancer using a previously created (22) breast cancer TMA. The intensity of Brk staining in each TMA sample was measured using Brk-specific antibodies; four arbitrary levels of Brk staining intensity were assigned (Fig. 1). The percentage carcinoma component for each sample was scored independently by two pathologists (Table 1), and the resulting data were subjected to statistical analysis as described in Methods. Brk protein was detected in 86% of the 250 TMA samples. The analysis revealed a strong correlation between the intensity of Brk staining and tumor grade as measured by the percentage carcinoma within each sample ($P < 0.0003$). Internal control samples (normal breast tissue) within the same TMA were uniformly Brk negative.

Brk is activated by ErbB receptors and promotes cellular proliferation. Previous reports (12, 19, 21) suggest that Brk signals downstream of EGFR (ErbB1). To further investigate Brk signaling downstream of ErbB receptors, we first examined a small panel of nontransformed mammary epithelial cells and breast cancer cell lines for coexpression of ErbB receptors and Brk. Cellular lysates from exponentially growing MCF-10A, HMEC, MCF-7, T47D, ZR-75-1, and SKBR3 cells were analyzed by Western blot with antibodies specific to ErbB1, ErbB2, ErbB3, ErbB4, Brk, and β-actin. Nontransformed mammary epithelial cells express high levels of ErbB1, low levels of ErbB2, and undetectable levels of ErbB3, ErbB4, and
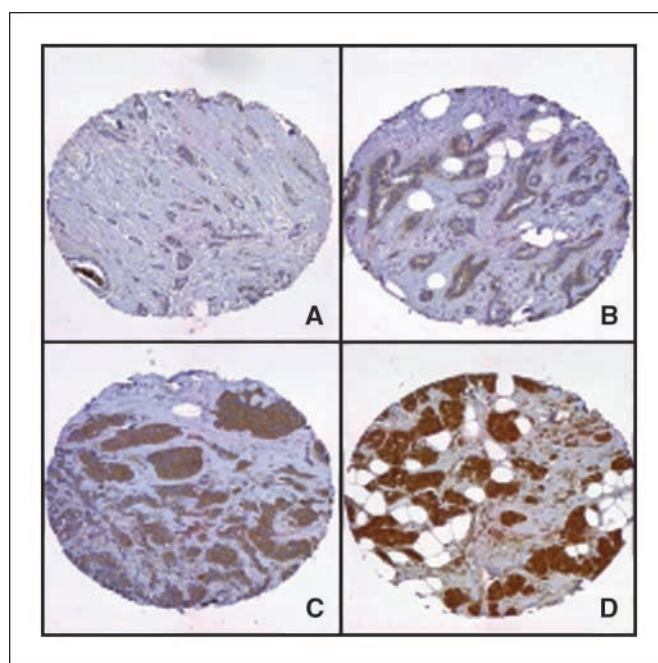

Figure 1. Intensity of Brk expression correlates with carcinoma component of invasive breast cancer. *A,* invasive ductal carcinoma with no expression of Brk (score 1). *B,* invasive ductal carcinoma invading in between fat cells with weak Brk expression (score 2). *C,* invasive ductal carcinoma forming nests of malignant cells with moderate Brk expression (score 3). *D,* invasive ductal carcinoma with strong expression of Brk protein (score 4). Magnification, ×100.

Table 1. Carcinoma content by Brk staining intensity

Brk staining intensity	No. samples	Carcinoma content (mean)	SD
1	34	28.97	28.25
2	63	31.11	22.62
3	107	41.50	23.87
4	46	44.67	25.35

NOTE: P value for linear trend is 0.0003.

Brk (Fig. 2*A*). All four breast cancer cell lines are Brk positive and express high levels of ErbB2, with varying levels of ErbB1, ErbB3, and/or ErbB4 (Fig. 2*A*). T47D breast cancer cells express Brk and all four ErbB receptors, making this cell line ideal to study Brk function in the context of ErbB receptor signaling.

Brk kinase activity, as measured by Brk autophosphorylation, is activated downstream of ErbB1 (EGFR) receptor signaling following EGF stimulation of COS1 cells transfected with Flag-tagged Brk (12) and siRNA depletion of Brk reduces EGF-induced phosphorylation of Sam68 (26). We wanted to determine if endogenous Brk was sensitive to additional ErbB ligands. We first developed an *in vitro* kinase assay using inducible stably expressed Brk and recombinant Sam68 as an *in vitro* Brk substrate. Using Brk-null MCF-10A cells as a starting point, we created multiple stable cell lines that inducibly express either wild-type (WT), kinase inactive (KM), or constitutively active (YF) Brk under the control of AP12967 dimerizer ligand (see Materials and Methods). MCF-10A cells stably expressing inducible WT and mutant Brk proteins were treated with either ethanol control or 2 nmol/L AP21967 (to induce Brk expression) for 24 h. Brk was then immunoprecipitated from whole-cell lysates using Brk-specific antibodies. Following extensive washing, Brk immunoprecipitates were incubated with a bacterially expressed, purified fragment of Sam68 in the presence of ATP and MgCl$_2$. Following kinase reactions, proteins were separated by SDS-PAGE, and tyrosine phosphorylated Brk and Sam68 were detected by Western blotting with anti–phosphorylated tyrosine antibodies (Fig. 2*B*). Following Brk induction, only cells expressing active WT or YF Brk, but not KM Brk, display tyrosine phosphorylated Brk and Sam68. Furthermore, no Brk activity was detected in Brk immunoprecipitates from uninduced (with AP21967) cells. Western blot controls indicated that equal levels of Brk protein were induced and immunoprecipitated in our assay system and that nonspecific kinase activities could not account for the *in vitro* phosphorylation of Sam68. These results repeated using the same assay done with purified recombinant Brk (data not shown) and suggest that Brk antibodies reliably and specifically immunoprecipitate Brk protein and that immunopurified active Brk undergoes autophosphorylation *in vitro* and phosphorylates recombinant Sam68.

Heregulins bind ErbB3 or ErbB4 receptors, which preferentially heterodimerize with ErbB2; ErbB2 receptors have no identified natural ligands. To determine if heregulin activates endogenous Brk kinase activity in breast cancer cells, T47D cells were deprived of serum (containing growth factors) for 24 h and then either left untreated or treated with 25 ng/mL heregulin for 5 to 60 min. As above, Brk was immunoprecipitated from whole-cell lysates using

Brk-specific antibodies and subjected to *in vitro* kinase assay (Fig. 2*C*). Rabbit IgG was included in some samples as a specificity control. Brk exhibited a low basal activity in untreated cells, consistent with expressed WT-Brk or YF-Brk in MCF-10A cells (Fig. 2*B*). However, relative to basal Brk activity, heregulin induced a rapid increase in Brk autophosphorylation within 5 min, and this was sustained throughout the 60-min time course. Additionally, Brk-induced *in vitro* phosphorylation of Sam68 increased between 15 and 30 min following heregulin treatment. Brk activity, as measured by phosphorylated Sam68, returned to basal levels by 60 min (Fig. 2*C*). Roughly equal levels of Brk were present in immunoprecipitation reactions; cell lysates indicate loading and input controls. Brk autophosphorylation and Sam68 phosphorylation are two independent readouts for Brk kinase activity. Brk autophosphorylation precedes efficient phosphorylation of Sam68 *in vitro*, suggesting that Brk autophosphorylation is an important activation step before substrate recognition; Brk may have different substrate specificities depending on its phosphorylation status. Supporting this idea, work from Qiu and Miller (27) suggests that Brk kinase activity is regulated by intramolecular interactions between Brk NH$_2$-terminal tyrosine residues and the Brk SH2 domain, and a polyproline-rich motif with the SH3 domain. Furthermore, the Brk SH3 domain seems to be important for substrate recognition (28). We also observed a modest increase in Brk kinase activity in response to EGF but not insulin-like growth factor (IGF) treatment of breast cancer cells (data not shown).

To determine the function of Brk in response to activation of ErbB signaling, we first knocked down Brk expression in T47D cells. Three distinct shRNA sequences targeting endogenous Brk and a nonspecific negative control shRNA sequence were subcloned into pSIREN RetroQ and retrovirus was prepared. Following infection, T47D cells were selected in 1 μg/mL puromycin and resistant cells were pooled. As shown in Fig. 2*D*, Brk protein levels were efficiently knocked down in two independent pools of cells selected for stable expression of Brk shRNA-B. Harvey and Crompton (20) showed that knockdown of Brk levels in breast cancer cell lines inhibits proliferation. To confirm these data and thus control for similar effects in our system, we did MTT cellular proliferation assays. Negative control shRNA and Brk shRNA T47D cells were plated in triplicate in standard growth medium. Twenty-four hours later, a day 0 time point was taken. Viable stained cells were quantified daily for 7 days and normalized to day 0 time points. Compared with cells expressing negative control shRNA, we observed a decrease in the relative cell number of the pools of cells expressing Brk shRNA starting at day 4 and continuing to day 7 (Fig. 2*E*). No significant increase in the number of nonviable cells was detected in 7-day Brk shRNA–expressing cultures relative to controls (data not shown), suggesting that Brk knockdown can moderately inhibit breast cancer cell proliferation, consistent with previous studies (20).

Brk promotes ERK5 and p38 activation. To investigate signaling pathways downstream of ErbB receptors that may be altered during Brk knockdown, serum-starved T47D cells expressing either negative control shRNA or Brk shRNA were stimulated with 25 ng/mL EGF or heregulin for 0 to 60 min. Whole-cell lysates were then analyzed by Western blot with antibodies specific for ERK5 and phosphorylated p38 mitogen-activated protein kinase (MAPK) and total p38 MAPK, Akt, ERK1/2 MAPKs, JNK, and Brk. Activated ERK5 was measured by Western blotting using a comparison of inactive or fast-migrating ERK5 relative to active or "upshifted" (slower mobility) phosphorylated ERK5 bands in
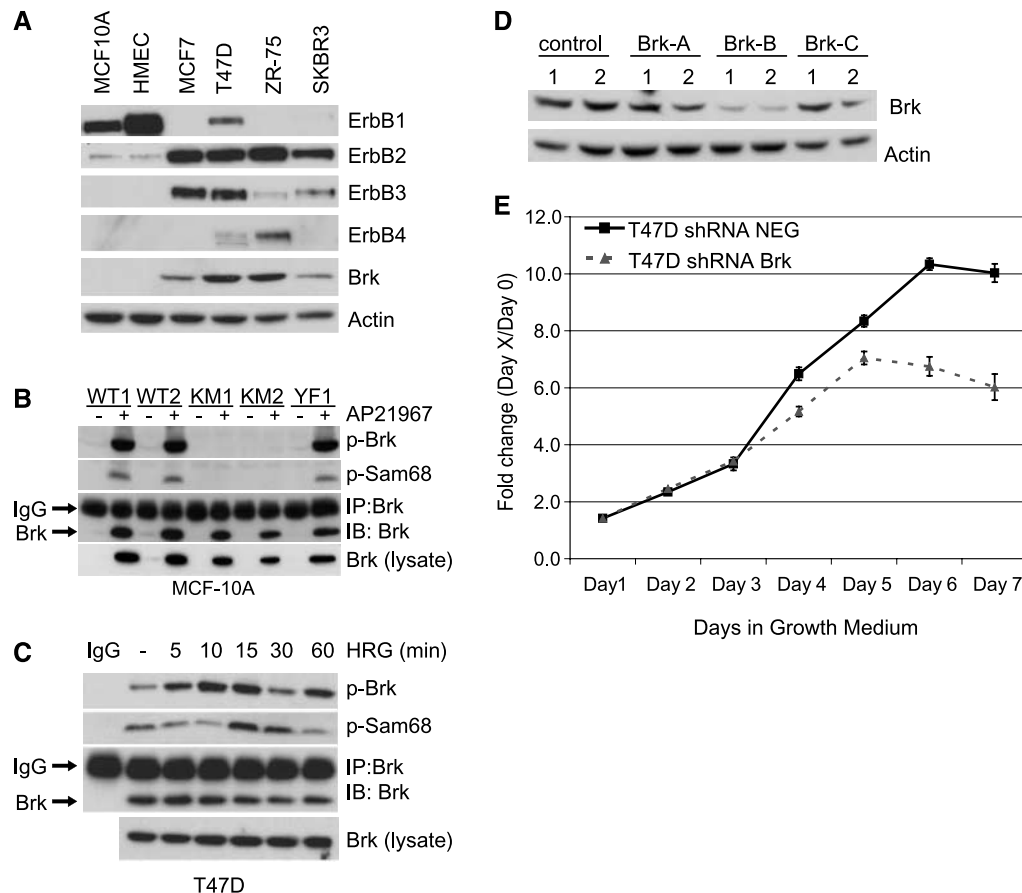
Figure 2. Brk is activated by ErbB receptors and promotes cellular proliferation. *A,* Western blot analysis for ErbB receptor and Brk expression in MCF-10A, HMEC, MCF-7, T47D, SKBR3, and ZR-75-1 cell lines. Equal amounts of protein from whole-cell lysates were loaded onto the gels and subjected to Western blotting using specific antibodies. *B, in vitro* Brk kinase assay in MCF-10A cells stably expressing inducible Brk proteins. Brk expression was induced (+) or uninduced (−) using AP21967 in multiple clones of MCF-10A cells stably expressing WT (*WT1* and *WT2*), kinase-inactive (*KM1* and *KM2*), or active (*YF*) Brk, and cell lysates were subjected to immunoprecipitation (*IP*) and *in vitro* kinase assay using purified recombinant Sam68 as a substrate (see Materials and Methods). Tyrosine phosphorylated Brk (*p-Brk*) and phosphorylated Sam68 (*p-Sam68*) were visualized using phosphorylated tyrosine-specific antibodies, and induced Brk in cell lysates and immunoprecipitates was detected using Brk-specific antibodies (*IB*). *C,* Brk kinase assay in T47D breast cancer cells. Serum-starved T47D cells were left untreated (−) or stimulated with heregulin (*HRG*) for 5 to 60 min and cell lysates were subjected to Brk immunoprecipitation and *in vitro* kinase assays as in (*B*). *D,* Western blot for Brk and β-actin in stable pools of T47D cells infected with retrovirus encoding Brk-specific shRNA (Brk-A, Brk-B, and Brk-C) or shRNA control. *E,* MTT cellular proliferation assay with T47D cells expressing either control shRNA (*NEG*) or Brk shRNA (Brk-B). Data are representative of three independent experiments.

SDS-PAGE gels. Complete activation of ERK5 results in the absence of the lower, faster-migrating (inactive) band and the appearance of the slower-migrating, upper band (29). As shown in Fig. 3*A,* EGF or heregulin induces activation of ERK5 at 10 to 30 min in control cells. However, we observed a decrease in activated ERK5 (upper band) at 10 min in cells treated with EGF and at 10, 15, and 30 min in response to heregulin in the cells expressing Brk shRNA relative to the cells expressing control shRNA. Western blotting with phosphorylated ERK5 antibodies also showed a decrease in ERK5 phosphorylation in Brk shRNA–expressing cells (see below; data not shown). Interestingly, activated p38 MAPK, identified with phosphorylation specific antibodies, was also greatly diminished in the Brk shRNA T47D cells at 10 and 15 min following either EGF or heregulin treatment. In a typical experiment, densitometry of bands indicated >90% decrease in the level of activated phosphorylated p38 in Brk shRNA–expressing T47D cells treated with EGF or heregulin relative to control cells. Akt, ERK1/2 MAPK, and JNK were activated to a similar level in both Brk shRNA and control

shRNA cells. In a similar set of experiments conducted in SKBR3 cells, we observed a similar decrease in ERK5 and p38 MAPK activation in response to heregulin following Brk knockdown (Fig. 3*B*), although ERK5 was inhibited to a lesser extent. These results were consistently obtained from at least three independently selected pools of cells; SKBR3 cells were consistently somewhat more resistant to Brk knockdown than T47D cells.

Next, we wanted to determine if the effect of Brk knockdown on p38 MAPK activation is specific to ErbB receptor signaling. Proinflammatory cytokines, such as interleukin-1β (IL-1β), activate p38 independently of ErbB receptors. Therefore, T47D cells expressing either control shRNA or Brk shRNA were stimulated with 5 ng/mL IL-1β for 0 to 60 min. Cellular lysates were then analyzed by Western blotting for phosphorylated and total p38 and Brk. IL-1β efficiently activated p38 in both the control and Brk shRNA T47D cells (Fig. 3*C*). A similar result was also observed in response to UV irradiation (data not shown), suggesting that Brk specifically promotes p38 MAPK activation downstream of ErbB

receptors. IκBα degradation was also examined to determine if Brk knockdown alters IL-1β–induced nuclear factor-κB (NF-κB) activation. IκBα was efficiently phosphorylated and degraded in IL-1β–treated control shRNA– and Brk shRNA–expressing T47D cells (Fig. 3C), suggesting that Brk does not mediate cytokine-induced NF-κB activation.

Brk shRNA inhibits heregulin-induced Rac activity. ErbB receptor activation results in the activation of numerous signal transduction cascades via recruitment of adapter molecules to the intracellular domains of ErbB receptor pairs, leading to the activation of small GTPases in the Ras superfamily. Rac is upstream of p38 (30) and EGF-induced activation of ERK5 is independent of Ras in mammary epithelial cells (29). To determine if Brk inputs to Rac activation in our system, T47D cells expressing control shRNA or Brk shRNA were deprived of growth factors by serum starvation for 48 h and then left either untreated (−) or treated with heregulin for 5 or 10 min. Whole-cell lysates were then incubated with GST-PAK(PBD) and extensively washed, and the amount of active GTP-bound Rac bound to PAK(PBD) in pulldowns

was determined by Western blot analysis with Rac-specific antibodies. Western blot analysis of cellular lysates with Brk and Rac antibodies was also done to control for protein loading and Brk expression. Brk knockdown in T47D cells blocks heregulin-induced activation of Rac relative to cells expressing control shRNA (Fig. 4). These results suggest that Brk acts downstream of ErbB receptors but upstream of Rac activation in response to heregulin. Densitometry comparing the amount of GTP-Rac/total Rac typically (n = 4) indicated a 90% and 64% decrease in GTP-Rac at 5 and 10 min, respectively, in independent pools of Brk shRNA–expressing cells.

Brk promotes p38-dependent activation of MEF2 transcription factors. One common downstream target of p38 MAPK and ERK5 is the MEF2C transcription factor. Interestingly, MEF2 transcription factors are well-characterized substrates for both p38 and ERK5 in muscle and neurons (31–33) but have not been implicated in breast cancer. We first examined MEF2C expression in breast cancer cell lines. Cellular lysates from exponentially growing MCF-10A, HMEC, MCF-7, T47D, ZR-75-1, and SKBR3 cells
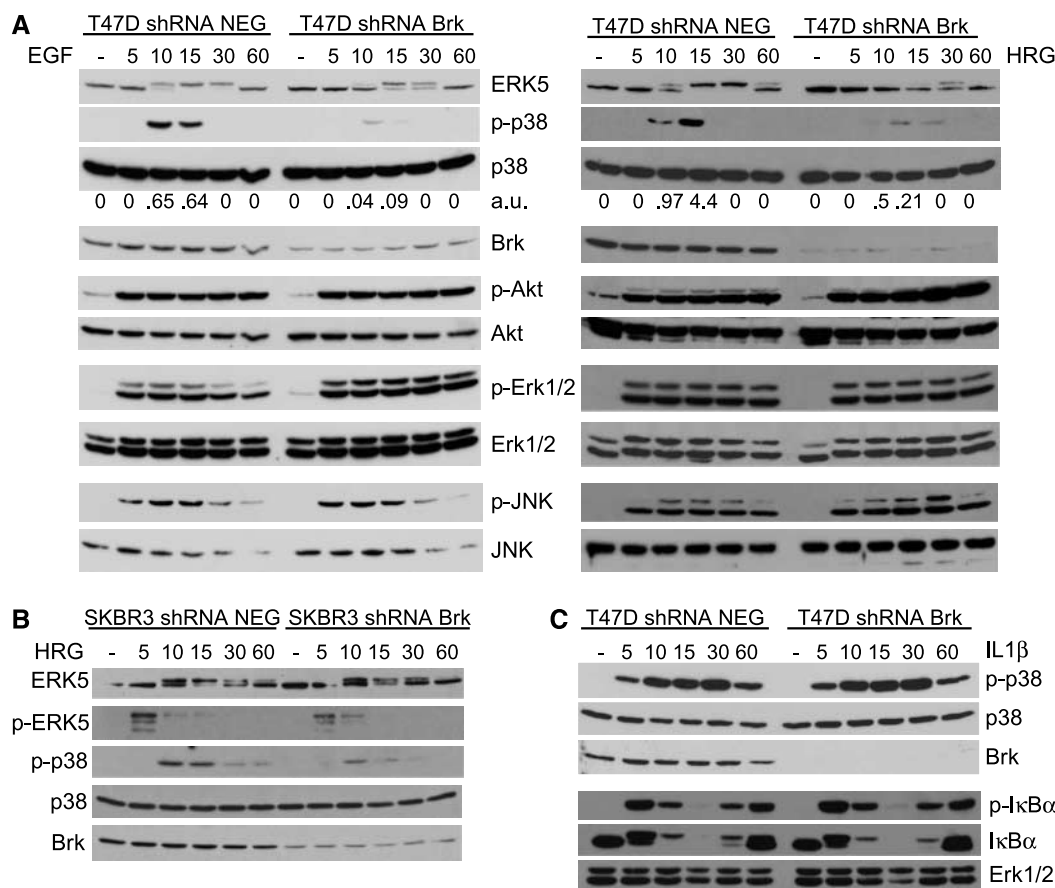

Figure 3. Brk promotes ERK5 and p38 activation. *A,* cellular lysates from T47D cells expressing control shRNA or Brk shRNA were treated with 25 ng/mL heregulin for 0, 5, 10, 15, 30, or 60 min and Western blotted with antibodies specific to ERK5 and phosphorylated and total p38 MAPK, Akt, ERK1/2 MAPKs, and JNK, and Brk. Densitometry results of phosphorylated p38/p38 are indicated below p38 Western blot in arbitrary units (*a.u.*). Percentage inhibition in phosphorylated p38 was determined by the equation 100 − [100 × (arbitrary units Brk shRNA / arbitrary units control shRNA)]. *B,* cellular lysates from SKBR3 cells expressing control shRNA or Brk shRNA treated with 25 ng/mL heregulin for 0, 5, 10, 15, 30, or 60 min were Western blotted with antibodies specific to Brk and phosphorylated and total ERK5 and p38 MAPK. Phosphorylated ERK5 antibodies recognize ERK rapidly phosphorylated on sites distinct from those inducing gel mobility upshifts observed with total ERK5 antibodies. *C,* Western blot analysis of phosphorylated p38 and total p38, Brk, phosphorylated IκBα and total IκBα, and total ERK1/2 from cellular lysates of T47D cells expressing either control or Brk shRNA treated with 5 ng/mL IL-1β for 0, 5, 10, 15, 30, and 60 min. Data are representative of at least three independent experiments in a minimum of three independently selected cell populations.
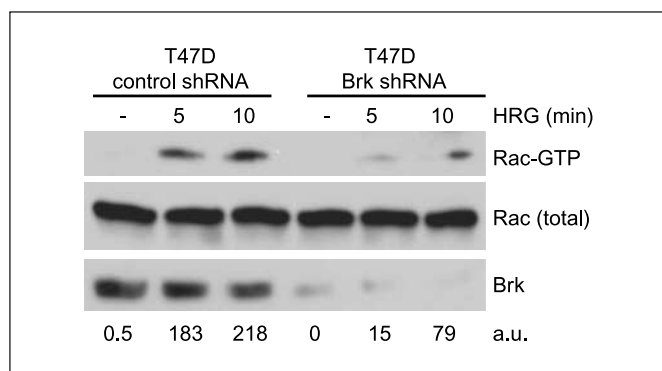
Figure 4. Brk shRNA inhibits heregulin-induced Rac activity. The amount of active Rac was determined from lysates of control and Brk shRNA T47D cells treated with 25 ng/mL heregulin for 0, 5, or 10 min. Cellular lysates were incubated with GST-PAK(PBD) and bound GTP-Rac was resolved by SDS-PAGE, and Western blots for Rac from the GST-pulldown and Rac and Brk from the cellular lysate were done. Densitometry results of GTP-Rac/Rac are indicated below Brk Western blot in arbitrary units. Percentage inhibition in GTP-Rac was determined by the equation 100 − [100 × (arbitrary units Brk shRNA / arbitrary units control shRNA)]. Data are representative of three independent experiments using independently selected cell populations.

were analyzed by Western blot with antibodies specific to MEF2C and Brk (Fig. 5*A*). We observed MEF2C expression in normal mammary epithelial cells and all breast cancer cell lines examined. Although serum has been shown to regulate MEF2 transcriptional activity, the effects of EGF and heregulin on MEF2 have not been examined. To determine if ErbB ligands and Brk expression activates the transcriptional activity of MEF2, we used a reporter construct containing three copies of a high-affinity MEF2 binding sequence from the desmin promoter linked to a minimal heat shock protein 70 promoter and inserted upstream of the luciferase reporter gene (34). To measure the ability of Brk to stimulate the transcriptional activity of endogenous MEF2, we first did luciferase assays in Brk-negative MEF2-positive HeLa cells. HeLa cells were plated and 24 h later transfected with 3× MEF2-luciferase and increasing concentrations of cDNA encoding WT-Brk and vector control for transfection normalization. As HeLa cells lack the full complement of ErbB receptors enabling heregulin responsiveness (i.e., do not express ErbB3 or ErbB4; data not shown), serum-starved (24 h) cells were either left untreated or treated with 1% FBS or 20 ng/mL EGF for 6 to 8 h. Whole-cell lysates were examined for luciferase and β-galactosidase activity as a control for transfection efficiency. As shown in Fig. 5*B*, MEF2-luciferase activity is induced by FBS and EGF in the absence of Brk. Additionally, Brk expression clearly increases the basal level of MEF2 transcriptional activity in a dose-dependent manner and further enhances FBS- and EGF-induced MEF2-luciferase activity. To determine if Brk-induced MEF2 transcriptional activity is dependent on p38 MAPK activation, HeLa cells were transfected with 3× MEF2-luciferase and either vector control or Brk cDNA. Following overnight serum starvation, cells were then pretreated for 30 min with either DMSO or 10 μmol/L SB203580 (p38 inhibitor) and then either left untreated or treated with 20 ng/mL EGF for 6 to 8 h. Again, EGF stimulation increased basal MEF2 transcriptional activity, and this was enhanced by Brk expression. The p38 MAPK inhibitor slightly reduced basal MEF2 activity. Notably, Brk-dependent activation of both basal and EGF-induced MEF2 transcriptional activity was blocked by inhibition of p38 MAPK signaling, suggesting that Brk-induced p38 MAPK activity is

a direct input to MEF2 activation (Fig. 5*C*). We next wanted to determine if heregulin stimulated MEF2 transcriptional activity in breast cancer cell lines and if Brk-dependent signaling to p38 is required. T47D and SKBR3 cells expressing either control or Brk shRNA were plated and the following day transfected with 3× MEF2-luciferase. Following 24 h of serum starvation, cells were pretreated for 30 min with either vehicle control (DMSO) or 10 μmol/L SB203580 and then either left untreated or treated with 25 ng/mL heregulin for 24 h. In T47D (Fig. 5*D, right*) and SKBR3 (Fig. 5*D, left*) shRNA control cells, heregulin increased MEF2-dependent luciferase activity. Furthermore, inhibition of p38 activity blocked heregulin-induced activity, suggesting that p38 activation is required for heregulin-induced MEF2-luciferase activity. Finally, T47D and SKBR3 cells expressing Brk shRNA clearly display reduced basal and heregulin-induced MEF2 transcriptional activity that was not further reduced by inhibition of p38 MAPK (Fig. 5*D*). These results suggest that Brk inputs to p38 MAPK–dependent activation of MEF2 transcription factors in breast cancer cells.

Brk promotes EGF- and heregulin-induced cyclin D1 expression. In addition to phosphorylation and activation of nuclear transcription factor substrates, ERK5 and p38 MAPKs have both been shown to promote increased cyclin D1 expression (35, 36). To determine if Brk is important for EGF- and heregulin-induced cyclin D1 expression in breast cancer cells, growth factor–deprived (serum starved) T47D cells expressing either control shRNA or Brk shRNA were either left untreated or stimulated with 25 ng/mL EGF or heregulin for 1, 3, or 6 h. Cellular lysates were analyzed by Western blotting with antibodies specific to cyclin D1 and Brk. Blots of total ERK1/2 (heregulin) or actin (EGF) were included as gel loading controls. In shRNA control cells, an increase in cyclin D1 protein expression was observed at 3 and 6 h after hormone treatment. Brk knockdown attenuated cyclin D1 expression in response to either EGF or heregulin, relative to controls (Fig. 6*A*). Additionally, as reported previously (36), pretreatment of T47D cells with 10 μmol/L SB203580 (p38 inhibitor) inhibited heregulin-induced cyclin D1 expression at 6 h following treatment (Fig. 6*B*), further showing the importance of Brk-induced p38 activation downstream of ErbB receptors.

Brk shRNA inhibits heregulin-induced migration. In addition to the established role of cyclin D1 in cell cycle progression as the partner of cyclin-dependent kinase (CDK) 4/CDK6 during G1-S transition and the direct regulation of transcriptional events related to cell transformation (37), recent reports suggest that cyclin D1 may also promote cellular migration (38, 39). Additionally, both p38 and ERK5 activation have been implicated in enhanced migration and metastasis (40, 41). To address the role of Brk in heregulin-induced breast cancer cell migration, we did Boyden chamber migration assays. For each Boyden chamber assay, control shRNA or Brk shRNA T47D cells were plated in the upper chamber in growth factor–free medium supplemented with 5 μg/mL collagen I. A polycarbonate membrane with 12 μmol/L pores separated the upper and lower cambers; lower chambers contained growth factor–free medium with 5 μg/mL collagen I with or without 25 ng/mL heregulin. Following cell plating, Boyden chambers were incubated for 6 h; the remaining cells in the upper chamber were removed, and the cells that migrated to the lower chamber were stained with HEMA3. The number of cells that migrated through each membrane was determined by counting five randomly chosen fields per well using a light microscope with a 10× objective. Heregulin induced a dramatic increase in T47D cell migration in control cells. However, heregulin-induced
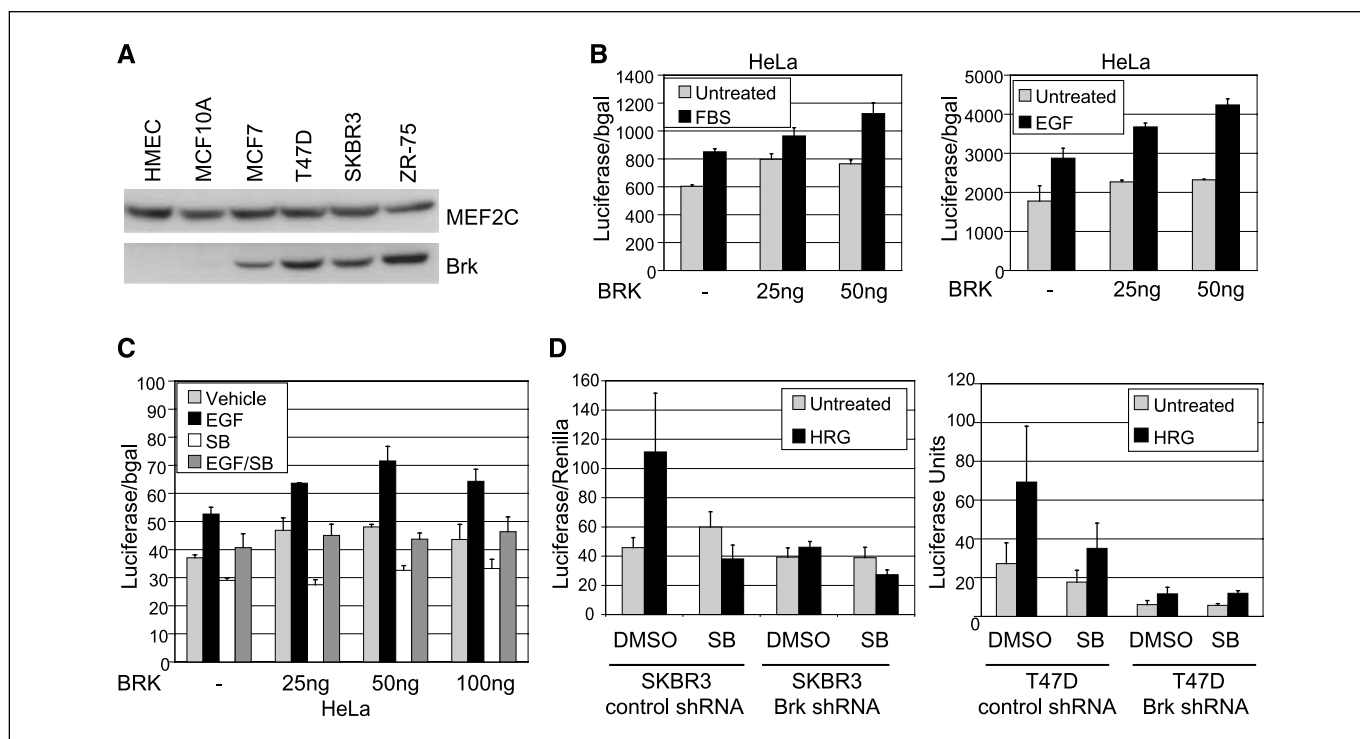
Figure 5. Brk promotes p38-dependent activation of MEF2 transcription factors. *A*, Western blot analysis for MEF2C and Brk expression in HMECs, MCF-10A, MCF-7, T47D, SKBR3, and ZR-75-1 cell lines. *B*, luciferase assays from HeLa cells transfected with 3× MEF2-luciferase and β-actin-LacZ reporters and increasing concentrations of Brk cDNA. HeLa cells were untreated or treated with 1% FBS (*left*) or 25 ng/mL EGF (*right*) for 6 to 8 h before cellular lysis. *C*, as in (*B*), luciferase assays were done in HeLa cells transfected with 3× MEF2-luciferase, β-actin-LacZ, and increasing concentrations of Brk cDNA. Cells were then pretreated for 30 min with either SB203580 (*SB*) or vehicle control (DMSO) and left untreated or treated with 25 ng/mL EGF for 6 to 8 h before cellular lysis. *D*, SKBR3 (*left*) and T47D (*right*) cells expressing control or Brk shRNA were transfected with 3× MEF2-luciferase and pRL-*Renilla* and then pretreated with either DMSO (vehicle) or SB203580 and left untreated or treated with 25 ng/mL heregulin for 24 h. Luciferase units were normalized to *Renilla*. For all luciferase assays, cells were plated in triplicate for each condition. Data are representative of at least three independent experiments.

migration was attenuated by >50% in Brk shRNA–expressing T47D cells compared with control cells (Fig. 7*A*). Similar results were observed with EGF-stimulated cell migration (data not shown). We did a similar set of experiments with T47D cells in the presence or absence of SB203580 to inhibit heregulin-induced p38 MAPK activation. T47D cells were incubated in growth factor–free medium supplemented with 5 μg/mL collagen I in the presence of 10 μmol/L SB203580 or DMSO (vehicle) for 30 min. Cells (1.5 × 10⁵) were then plated in the presence or absence of SB203580 in the upper chamber, whereas the lower chamber contained growth factor–free medium supplemented with 5 μg/mL collagen I alone or with 25 ng/mL heregulin. After 6 h, the number of the cells that migrated to the lower chamber was measured as above. Similar to our results with Brk shRNA–expressing cells, the p38 inhibitor reduced heregulin-induced T47D cell migration by >50% (Fig. 7*B*). Taken together, these data suggest that Brk promotes ErbB receptor activation of p38 MAPK, which potentiates heregulin-induced breast cancer cell migration.

Next, we wanted to determine if heregulin-induced cyclin D1 expression is required for migration of T47D cells. Cyclin D1–specific and control siRNAs were transiently transfected into T47D cells, and 48 h later, cells were trypsinized and assayed for migration in Boyden chambers as described above. Cyclin D1 siRNA inhibited basal and heregulin-induced migration of T47D cells (Fig. 7*C*). However, the fold increase in heregulin-induced migration was not significantly altered in response to cyclin D1 knockdown. These results suggest that cyclin D1 expression

contributes to the basal migration phenotype of T47D cells and that heregulin-induced, Brk-dependent up-regulation of cyclin D1 is important but not sufficient for breast cancer cell migration.

Discussion

Although recent reports have identified novel Brk substrates and potential signaling pathways regulated upstream of Brk activation (12, 19, 21), the function of Brk in breast cancer development or progression remains understudied and unclear. Herein, we sought to better understand the contribution of Brk-dependent downstream pathways in response to ErbB-initiated signaling events known to be important for breast cancer biology. We show Brk expression in up to 86% of 250 invasive ductal breast carcinomas, the largest cohort sampled to date (Fig. 1). Additionally, we report for the first time that Brk is activated by heregulin (Fig. 2) and promotes EGF- and heregulin-induced ERK5 and p38 MAPK activation (Fig. 3), activation of MEF2 transcription factors (Fig. 5), and increased expression of cyclin D1 in breast cancer cells (Fig. 6). Furthermore, we show that activation of Brk-dependent downstream pathways (i.e., p38 MAPK) is essential for EGF- and heregulin-induced migration of breast cancer cells (Fig. 7).

Our results suggest that Brk mediates ErbB signaling upstream of Rac, p38 MAPK, and ERK5. In our model, activation of these pathways is important for regulation of MEF2 and cyclin D1 and cell migration induced by ErbB ligands. Importantly, Rac, p38, ERK5, and cyclin D1 have all been implicated in breast cancer

biology. Rac is an important mediator of heregulin-induced cell growth and migration of T47D and MCF-7 breast cancer cells (42). In their report, Yang et al. (42) showed that ErbB1, ErbB2, and ErbB3 were required for heregulin-induced Rac activation, and Rac-RNAi or DN-Rac inhibited both heregulin-induced proliferation and migration. Consistent with our results herein, a link between Brk, Rac activation, and migration has been described previously (12). Chen et al. (12) showed that Brk phosphorylation of paxillin leads to CrkII-dependent activation of Rac-1; phosphorylation site-mutant paxillin blocked Brk-induced invasion of A431 and MDA-MB-231 cells.

Our studies place Brk upstream of p38 MAPK, a mediator of MEF2 activation, cyclin D1 up-regulation, and increased cell migration in response to ErbB signaling (Figs. 3, 6, and 7). In breast cancer cells and other model systems, p38 MAPK is relatively understudied compared with classic (p42/p44) MAPKs. p38 MAPK was initially found to be activated under cellular stress and by proinflammatory cytokines; however, p38 is also well activated by growth factor signaling pathways. Increasing reports support the hypothesis that p38 activation may enhance breast cancer cell survival (43) and metastasis/invasion (44) and that phosphorylation (activation) of p38 may serve as a prognostic indicator for shortened progression-free survival (45). Furthermore, Gutierrez et al. (46) found that high levels of active phosphorylated p38 MAPK strongly correlated with up-regulated HER2 (c-ErbB2) and increased tamoxifen resistance in clinical specimens and using the MCF-7 xenograft model of breast tumor progression. Similar to data presented here (Fig. 6), it has been reported that p38
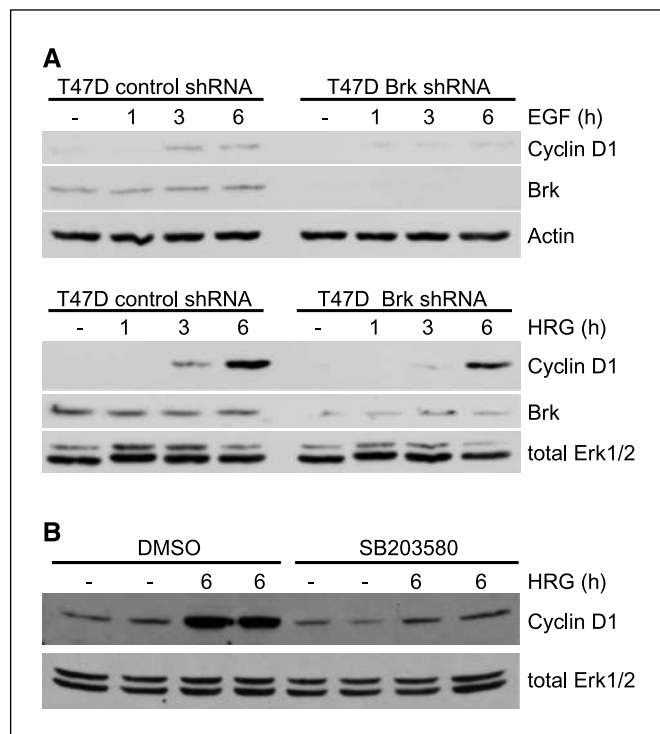

Figure 6. Brk promotes EGF- and heregulin-induced cyclin D1 expression. *A,* cellular lysates from T47D cells expressing control or Brk shRNA treated with 25 ng/mL EGF or heregulin for 0, 1, 3, or 6 h were Western blotted with antibodies specific to cyclin D1, Brk, actin (*EGF*), or ERK1/2 (*HRG*). *B,* cyclin D1 Western blot analysis from duplicate cultures of T47D cells pretreated with DMSO (vehicle) or SB203580 for 30 min and then untreated or treated with heregulin for 6 h.
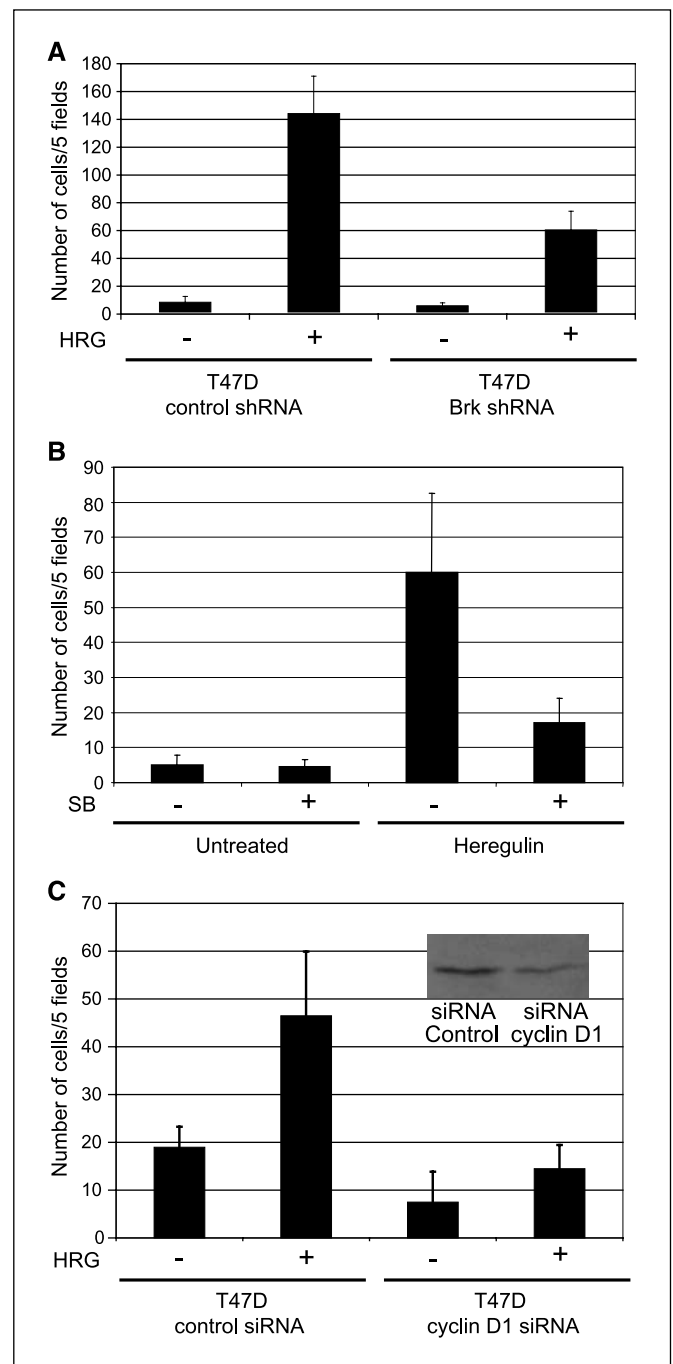

Figure 7. Brk shRNA inhibits heregulin-induced migration. *A,* Boyden chamber migration assay with control and Brk shRNA–expressing T47D cells. Lower chambers contained MEM with 5 μg/mL collagen I, with (+) or without (−) 25 ng/mL heregulin. Upper chambers contained 1.5 × 10⁵ cells in MEM with 5 μg/mL collagen I. *B,* Boyden chamber migration assays were done as above using T47D cells pretreated with either vehicle control (−) or SB203580 (+) for 30 min before plating in upper chambers. *C,* Boyden chamber assays done as described in (*A*) with the exception that control shRNA T47D cells were transiently transfected with cyclin D1 siRNA 48 h before migration assays and cells were allowed to migrate for 16 h.

activation promotes heregulin-induced cell cycle progression and expression of D type cyclins (36). Additionally, p38 activation mediates IGF-induced migration of breast cancer cells (41). Of note, we did not observe increased Brk activity in response to IGF-I treatment of breast cancer cells (data not shown).

ERK5 is the newest and least well-defined member of the MAPK superfamily. Like the other MAPK members, ERK5 is activated by growth factors and stress stimuli, including serum, EGF, oxidative stress, and UV irradiation. Deregulation of ERK5 signaling is associated with numerous types of human cancers; activated ERK5 is correlated with increased metastatic potential of prostate cancer cells, sustained malignant growth of mammary carcinomas, and chemoresistance of breast cancer cells (47). The ERK5 pathway [MAPK kinase kinase 2 (MEKK2)/MEKK3/MEK5/ERK5] is constitutively active in breast cancer cells overexpressing ErbB2 receptors, and blockade of ERK5 signaling resulted in inhibition of breast cancer cell growth (48). Recently, heregulin was shown to stimulate ERK5 activation (49). A few ERK5 targets have been identified. These include additional signaling molecules (serum- and glucocorticoid-inducible kinase), structural proteins (connexin 43, a gap junction protein), and transcription factors of the MEF2 and Sap1a (Ets domain) families. Notably, activation of MEF2 family members by ERK5 is critical for mediating growth factor–induced cell survival in endothelial cells. MEF2 also regulates serum-inducible *c-jun* expression, leading to cell cycle progression (50). Additionally, ERK5 is an independent (from ERK1/2) input to cyclin D1 transcriptional up-regulation, perhaps acting via MEF2C-dependent up-regulation and recruitment of c-jun to the cyclin D1 promoter (35). These reports indicate that ERK5 signaling contributes to breast cancer progression. Data presented here indicate that Brk enhances EGF- and heregulin-induced ERK5 activation (Fig. 3), providing further evidence for the linkage of Brk and ERK5 downstream of ErbB signaling, relevant to breast cancer biology. Our studies suggest a dominant role for p38 MAPK in heregulin-induced signaling downstream of Brk activation. The relative contribution of ERK5 activation in EGF- and heregulin-induced cyclin D1 expression, MEF2 transcriptional activity, and breast cancer cell migration is of great interest and is the subject of a separate study.

Of note, we did not detect alterations in ErbB receptor activation of Akt following Brk knockdown in breast cancer cells (Fig. 3). We reported previously that Brk expression can limit basal Akt kinase activity in COS cells and keratinocytes (15). Brk and Akt copurified in immunoprecipitated complexes that dissociated on EGF treatment; WT-Brk, but not KM-Brk, inhibited Akt in purified complexes. Our findings are in good agreement with recently published data from Sik knockout mice, which display an increase in Akt activity in the small intestine (17). Taken together, these reports reveal important differences in Brk signaling between normal (gut and skin epithelial cells) and neoplastic (breast cancer) contexts. Proto-oncogenic Brk may constrain the Akt signaling pathway in cells where Brk is appropriately expressed, whereas oncogenic (i.e., overexpressed) Brk may inappropriately drive p38 and ERK5 signaling leading to increased cell proliferation and migration in cancer cells.

Cyclin D1 is an important mediator of cell cycle progression and tumorigenesis and an integral component of the cyclin D/CDK4 kinase complex, which targets the retinoblastoma protein (Rb). Phosphorylation of Rb leads to the release of E2F family proteins, which in turn activate gene transcription to regulate transition through the G_1 phase of the cell cycle. *Cyclin D1* gene amplification has been observed in 20% of breast tumors, whereas >50% overexpress cyclin D1 protein. Cyclin D1 transgenic mice develop breast cancer (51), and targeted deletion of cyclin D1 from the mouse mammary gland suppresses Neu(ErbB2)- and Ras-induced tumorigenesis (52). Interestingly, both p38 and ERK5 have been shown to regulate cyclin D1 protein levels. Specifically, p38 inhibitors have been shown to inhibit heregulin-induced expression of cyclin D1 and progression into S phase in breast cancer cell lines (36). Notably, inhibition of cyclin D1 expression following Brk knockdown or p38 MAPK inhibition did not map to cyclin D1 mRNA expression,[4] suggesting that Brk signaling to p38 MAPK or ERK5 may act to stabilize cyclin D1 protein.

In addition to the role of cyclin D1 in the cell cycle, recent reports suggest cyclin D1 may be important for cellular migration (38, 39). Bone marrow macrophages from cyclin D1–deficient mice display reduced migration in response to wounding, cytokine-mediated chemotaxis, and transendothelial migration (39). Fu et al. (38) showed that cyclin D1–deficient mouse embryo fibroblasts display decreased migration in wound healing and Transwell migration assays. Interestingly, cyclin D1 seems to act similarly in T47D cells, perhaps as a mediator of basal migration responses (Fig. 7C). Heregulin significantly increases the migration of T47D cells, while at the same time inducing cyclin D1 expression. Knockdown of cyclin D1 expression clearly inhibited basal T47D cell migration and partially reduced cell migration in response to heregulin (Fig. 7C). Both cyclin D1 expression and migration are dependent on heregulin-induced p38 MAPK activation (Figs. 6 and 7).

In addition to cell proliferation (discussed above), MEF2 transcription factors also regulate cellular differentiation (32). There are four vertebrate MEF2 genes (*MEF2A, MEF2B, MEF2C*, and *MEF2D*). MEF2 proteins have a highly conserved NH_2-terminal domain that mediates dimerization, cofactor binding, and DNA binding, whereas the COOH-terminal domain is required for transcriptional activation. MEF2 has mostly been studied in muscle and more recently in neurons. However, like Brk in skin cells, muscle MEF2 is exquisitely sensitive to calcium-dependent signaling pathways (32). Additionally, MEF2 is well activated by ERK5 and p38 MAPKs via phosphorylation events within the NH_2-terminal and COOH-terminal domains, respectively (32, 33). We show here that Brk primarily signals via activation of ERK5 and p38 MAPK in breast cancer cells. Additionally, we present here, for the first time, that (*a*) MEF2C is expressed in normal mammary epithelial cells and breast cancer cell lines, (*b*) EGF and heregulin activate Brk- and p38-dependent MEF2-luciferase activity, (*c*) Brk overexpression enhances MEF2-luciferase activity, and (*d*) Brk shRNA inhibits heregulin-induced MEF2-luciferase activity (Fig. 5). Interestingly, synthetic derivatives of distamycin A, an anticancer agent that targets the ability of MEF2 proteins to interact with DNA (53), are currently in phase I clinical trials (54). Future studies are aimed at determining which MEF2 family members are expressed in a majority of breast cancer cells, how they may contribute to breast cancer biology, and if they are key targets of Brk-regulated signaling pathways.

ErbB1 and ErbB2 receptors have proven to be efficacious targets for the treatment of cancer. In women with ErbB2-positive metastatic breast cancer, Herceptin has been shown to significantly increase disease-free survival and overall survival (55). Unfortunately, only ∼30% of ErbB2-positive breast cancer patients respond to Herceptin therapy. This has prompted scientists to identify additional factors and pathways that may be targeted in addition to ErbB2. These studies support further investigation of Brk as a potential target for the treatment of advanced ErbB2-positive breast cancers. Additionally, our data underscore the importance of p38 MAPK and ERK5 in breast cancer signaling mechanisms.

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[4] E.J. Faivre and C.A. Lange, unpublished results.

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EDUCATION

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RESEARCH EXPERIENCE

July 2005-present	University of Minnesota, Twin Cities Microbiology, Immunology and Cancer Biology Ph.D. Program **Research Project:** Breast tumor kinase (Brk/PTK6) mediated signaling and phenotypes in breast cancer cells **Mentor:** Carol A. Lange, Ph.D. **Contributions:** Engineered Brk/PTK6 knockdown breast cancer cell lines that were used to investigate Brk-dependent downstream responses to heregulin treatment; used Brk transgenic mouse model to investigate signaling events in involuting mammary glands and in tumorigenesis
Sept. 2003-June 2005	National Institutes of Health, National Cancer Institute Cell and Cancer Biology Branch, Rockville, MD **Research Project**: Investigating Ras-related proteins responsible for cellular responses to farnesyl transferase inhibitors (FTIs) **Mentor:** Geoffrey J. Clark, Ph.D. **Contributions:** Performed validation of a yeast-two-hybrid assay for novel effectors of the Ras-related proteins Ras-like inhibitor of growth (Rig) and Noey2/Arhi; investigated chemotherapeutic treatments *in vitro* that suggested mTOR signaling as a Ras-independent mechanism for growth in FTI resistant cancer cells

May 2002-Dec. 2002	US Department of Agriculture, Agricultural Research Service
	National Swine Research and Information Center, Ames, IA
	Research Project: Microbial ecology in biofilm formation
	Mentor: Kevin L. Anderson, Ph.D.
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AWARDS AND FELLOWSHIPS

09/2006-10/2009	Department of Defense Breast Cancer Research Program Pre-doctoral Fellowship (Award# BC061473, $97,200)
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PUBLICATIONS AND PRESENTATIONS

Ostrander JH, Daniel AR, Lofgren KA, Lange CA. Breast Tumor Kinase (Brk/PTK6) Regulates Heregulin-Induced Activation of Erk5 and p38 MAP Kinases in Breast Cancer Cells. Cancer Research 67(9): 4199-4209, 2007.

Lofgren KA, Lange CA. Brk Mediated Signaling and Mammary Gland Biology Relevant to Breast Cancer. 2007 Gordon Research Conference: Mammary Gland Biology, Newport, RI, June 2007. *Oral Presentation.*

Lofgren KA, Ostrander JH, Vanasek T, Qiu M, Lange CA. Breast Tumor Kinase Mediates Signaling and Biology Relevant to Breast Cancer Tumorigenesis and Progression. University of Minnesota Medical Research Day, June 2007. *Poster Presentation.*

Lofgren KA, Ostrander JH, Qiu M, Vanasek T, Lange CA. Breast Tumor Kinase Influences Survival Signals and Phenotypes in Breast Cancer Cells. MICaB Kick-off poster session, University of Minnesota, August 2006. *Poster Presentation.*

Lofgren KA, Ellis CA, Bell AB, Leaner VD, Birrer MJ, Ramchandran RR, and Clark GJ. Identification and Characterization of Rig2: A Novel Member of the Ras-Related Tumor Suppressor Subfamily. 4th Annual National Cancer Institute Fellows and Young Investigators Retreat, Williamsburg, VA, April 2004. *Poster Presentation.*

<u>Lofgren KA</u>, Ellis CA, Bell AB, Leaner VD, Birrer MJ, Ramchandran RR, and Clark GJ. Identification and Characterization of Rig2: A Novel Member of the Ras-Related Tumor Suppressor Subfamily. 20th Annual Oncogene Meeting, Frederick, MD, June 2004. *Poster Presentation.*

TEACHING

6/2007-7/2007	University of Minnesota Comparative Molecular Biosciences Department CMB 5335: Molecular Biotechnology Workshop Graduate Teaching Assistant Coordinator: Pam Skinner, Ph.D. -Prepared chemical and biological reagents for lab instruction -Organized classroom layout/logistics for the workshop -Performed exercises concurrently with students for quality control -Prepared demonstration exercises
1/2007-6/2007	University of Minnesota, Microbiology Department Microbiology 3301: Biology of Microorganisms Lab Graduate Teaching Assistant Coordinator: Christian D. Mohr, Ph.D. -Prepared lab lectures and quizzes weekly -Proctored exam sessions for lecture section of Micb 3301 -Created grading rubrics for weekly lab assignments
8/2001-5/2002	Iowa State University, Biology Department Biology 301L: Principles of Genetics Laboratory Undergraduate Teaching Assistant Coordinator: Claudia Lemper, M.S. -Prepared laboratory reagents and materials for class use -Prepared two lectures and two quizzes per semester -Assisted Graduate Teaching Assistant with questions during lab